STRUCTURAL RESPONSES OF AMOEBAE TO THE INJECTION OF HETEROLOGOUS CYTOPLASM

CHARLES J. FLICKINGER, GRAHAM A. READ AND ELLEN M. KABANA

Department of Anatomy, University of Virginia School of Medicine, Charlottesville, Virginia, U.S.A.

SUMMARY

Responses to the introduction of heterologous cytoplasm and the fate of foreign organelles were investigated in amoebae. Heterologous cytoplasm was transferred by microinjection from Pelomyxa carolinensis to Amoeba discoïdes. In control experiments, homologous cytoplasm was transferred from one A. discoïdes to another. Recipient cells were observed by light microscopy, and samples were prepared for ultrastructural study at intervals between 15 min and 3 days after operation. Recipients of heterologous cytoplasm showed two main responses. First, about 40% recipients expelled small amounts of cytoplasm by a blebbing process within 30 min after injection. Second, organelles were segregated and broken down in membrane-bounded cytoplasmic vacuoles between 6 h and 2 days after operation. Acid phosphatase reaction product was observed in these vacuoles along with altered organelles. Use of electron-dense thoria particles to mark donor cells demonstrated the presence of injected cytoplasm in the vacuoles. In contrast, when amoebae were injected with homologous cytoplasm, none was expelled and vacuoles containing degenerating organelles were uncommon. The survival rate and general appearance of recipients of heterologous cytoplasm were much poorer than those of homologous recipients, and most of the former died by 1 week after operation. It is postulated that amoebae are capable of recognizing heterologous organelles introduced into the cytoplasm and that they respond by expulsion and/or destruction of the foreign cellular components. The previously described lethal effect of heterologous cytoplasm was confirmed.

INTRODUCTION

The degree to which parts of different cells are compatible and how cells recognize and respond to foreign cellular components are basic questions in cell biology. Amoebae are useful in attempting to answer these questions because they can be manipulated microsurgically (Jeon & Danielli, 1971; Jeon, Lorch & Danielli, 1970). Experiments on compatibility of various combinations of nucleus and cytoplasm in hybrid amoebae and in heterokaryons formed by nuclear transplantation have demonstrated that a strain-specific incompatibility exists (reviewed by Jeon & Danielli, 1971; Yudin, 1973; Jeon & Lorch, 1973). For example, most hybrid cells with nucleus and cytoplasm from different strains of uninucleate amoebae are viable for a limited time and only a small fraction of them are able to form clones (Jeon, 1969; Goldstein, 1970; Jeon & Danielli, 1971). Similarly, in heterokaryons, one nucleus may exercise a lethal, mitosis-inhibiting effect upon the other nucleus (Jeon & Lorch, 1969; Yudin, 1973). On the other hand, the small proportion of long-term stable hybrid amoebae in which nucleus and cytoplasm are compatible has been useful to study the
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respective roles of nucleus and cytoplasm in determining cellular traits (see reviews by Jeon & Danielli, 1971; Hawkins, 1973).

Transfer of cytoplasm by microfusion (Daniels, 1962) or microinjection (Hawkins & Cole, 1965; Jeon & Lorch, 1970, 1971; Cameron & Hawkins, 1976) has shown that amoebae tolerate homologous cytoplasm well, but that introduction of heterologous cytoplasm usually leads within several days to death without cell division. This may be attributed to a lethal and antimitotic factor, which is present in the postmicrosomal supernatant fraction of homogenized amoebae (Jeon & Lorch, 1970; Cameron & Hawkins, 1976) and is capable of inhibiting RNA synthesis in cells of a different strain (Jeon & Lorch, 1971; Cameron & Hawkins, 1976). There is evidence that a high-molecular-weight protein is involved (Jeon & Lorch, 1971), although some traits in amoebae are known to be transferred in RNA-containing cytoplasmic fractions (Hawkins, 1969, 1973).

In the present study, Amoeba discoides were observed by light and electron microscopy following the injection of heterologous cytoplasm from the giant amoeba Pelomyxa carolinensis. Objectives were to determine the nature and extent of structural responses to foreign cytoplasm and the fate of heterologous organelles. The results indicate that some injected cytoplasm is rapidly expelled but that other organelles are segregated and degenerate within membrane-bounded spaces in the host cytoplasm. By comparison, the injection of homologous cytoplasm leads to few alterations.

MATERIALS AND METHODS

Cultures of Amoeba discoides, originally obtained from Dr K. W. Jeon, University of Tennessee, Knoxville, were maintained in Prescott's amoeba medium (Prescott & Carrier, 1964) with daily feedings of washed Tetrahymena. Cultures of Pelomyxa carolinensis were purchased from Carolina Biological Supply Co., Burlington, N.C. and were maintained in Chalkley's medium (Chalkley, 1930) with a boiled rice grain, hay, and daily feedings of Tetrahymena. Cultures were starved for 1 or 2 days prior to use. Pelomyxa carolinensis were used as donors of heterologous cytoplasm for Amoeba discoides recipients. In experiments on transfer of homologous cytoplasm, A. discoides served as both donors and recipients. Transfer of cytoplasm was carried out with an apparatus previously used for microinjection of radioactive materials into individual amoebae (Flickinger, 1974a), consisting of a 0.2-ml microsyringe (Gilmont Instruments, Great Neck, N.Y.) connected through polyethylene tubing to a glass micropipette (formed with a DKI Model 700C vertical pipette puller, David Kopf Instruments, Tunjunga, California). Using a braking pipette, amoebae were placed in a row on the surface of an agar-coated glass slide, and the excess medium was withdrawn flattening the cells on the surface (Jeon, 1970). Under a compound microscope at a magnification of 180 times, cytoplasm was withdrawn from a donor and injected into the recipients. The volume of cytoplasm injected was estimated to be roughly 0.1-0.3 x 10^6 μm³, or about 10% of the volume of recipient cells. After injection, recipients were transferred to a watchglass that contained amoeba medium.

Living injected amoebae were studied with a Zeiss stereomicroscope. Injected cells were fixed for electron microscopy at the following intervals after the operation: 15 min; 2, 6, and 12 h; and 1, 2, and 3 days. Samples of unmanipulated amoebae from the cultures of donors and recipients were also prepared for ultrastructural study. The cells were immersed for 1 h in Karnovsky's fixative (Karnovsky, 1965), rinsed overnight in 0.05 M cacodylate buffer pH 7.3, and embedded in a cube of agar to prevent their dispersion (Flickinger, 1969). The specimens were postfixed in 1% OsO₄ in 0.1 M cacodylate buffer pH 7.3, dehydrated in a graded series of ethanols followed by propylene oxide, and embedded in Araldite. Thick sections for light micro-
Cytoplasmic injection in amoebae

Scoppy were cut with glass knives and stained with toluidine blue. Silver to pale gold thin sections were cut with a diamond knife on a Porter-Blum MT-1 or MT-2B ultramicrotome, mounted on uncoated copper grids, and stained with uranyl acetate and lead citrate. The preparations were studied with a Philips EM-300 or AEI EM-801 electron microscope. The numbers of injected cells for ultrastructural study were limited to approximately 30–50 amoebae per sample by the necessity to perform time-consuming microsurgical manipulations. In experiments on the transfer of heterologous cytoplasm, two or more samples were studied ultrastructurally at each interval.

For localization of acid phosphatase activity, normal amoebae and recipients of heterologous cytoplasm 6 h after injection were fixed for 30 min in Karnovsky’s fluid and rinsed overnight in distilled water. They were incubated for 45 min at 37 °C in a medium consisting of 1 vol. of 1.25 % sodium β-glycerolphosphate (Sigma), 1 vol. of Tris-maleate buffer pH 5.0, 2 vol. of 0.2 % lead nitrate, and 1 vol. of distilled water (Barka & Anderson, 1962; Wise & Flickinger, 1970). Control cells were incubated in medium lacking the substrate. After incubation, the samples were rinsed successively in distilled water, 1 % acetic acid, and distilled water, 5 min each. The cells were then postfixed and embedded as usual.

In other experiments, the donor cytoplasm was labelled with electron-dense thorium particles. Donor amoebae were immersed for 30 min in a mixture of 0.4 ml of 2 % thorium solution (Polysciences, Inc., Warrington, Pa) and 0.6 ml of amoeba medium at pH 4.4. The cells were then rinsed, placed in normal amoeba medium, and used within the next 1–2 h as cytoplasm donors. Recipient cells were fixed 6 h after injection of cytoplasm.

RESULTS

Light-microscopic observations of living cells

During the operation, many granules of varying size were visible with the light microscope in the donor cytoplasm. These evidently represented a variety of structures more clearly identifiable in the electron microscope as crystals and organelles such as mitochondria and Golgi bodies. When heterologous cytoplasm from P. carolinensis was injected into an A. discoides, some granules immediately dispersed and mixed with similar components of the host. Continuous observation with the stereomicroscope, however, revealed that some cytoplasm, including granular donor cytoplasm that had failed to disperse from the injection site, was expelled from one-third to one-half of the recipients during the next 20–30 min by formation and pinching-off of a dome-shaped elevation, or bleb. In some cases, this was followed by lysis of the host cell, while in others the injected amoeba survived this process.

Immediately following the injection of heterologous cytoplasm the recipient cells were round and did not attach to the substrate. Over the next 5–6 h some of the recipient amoebae extended short pseudopods, attached to the substrate, and became motile. The round configuration was similar to that commonly assumed by these cells under deleterious conditions, while the extended motile forms resembled normal amoebae. Table 1 shows the results of an experiment in which injected amoebae were observed over a period of several days. Until the third day after the operation, one-half or more of the cells that had received heterologous cytoplasm were round. The amoebae then appeared transiently to undergo an improvement in their general condition as the proportion of round cells dropped through the sixth day postoperatively. This was followed, however, by a sharp decline in total number of cells as most of those injected with heterologous cytoplasm died.
Table 1. The appearance of amoebae after injection of heterologous or homologous cytoplasm

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Round cells did not attach to the substrate and were not motile. Extended cells had the typical polymorphous shape of normal amoebae, attached to the substrate, and were motile.

When cytoplasm from one *A. discoides* was injected into another amoeba of the same species, no cytoplasm was expelled. Within minutes many recipients attached to the substrate and became motile. On the days following the operation, in most cases less than 20% of the cells were round while the remainder were extended, motile, and appeared healthy. A decline in the total number of cells was observed over several days, but this was less severe than in the case of cells that received heterologous cytoplasm. Perhaps starvation contributed to the decline since *Tetrahymena* were added very sparingly to both groups of cells to avoid the deleterious effects of over-feeding on those that had received heterologous cytoplasm.

**Ultrastructural observations**

*Normal amoebae.* The fine structure of unmanipulated *P. carolinensis* and *A. discoides* conformed to previously published descriptions of normally amoebae (Daniels, 1973; Flickinger, 1973a, 1974b). Briefly, the rough endoplasmic reticulum consisted of irregularly shaped anastomosing cisternae and vesicles. Multiple Golgi bodies composed of a stack of smooth cisternae and associated vesicles were scattered throughout the cytoplasm. Mitochondria of both species presented circular and elongated profiles with tubular cristae. Many of those of *P. carolinensis* attained a large size, up to ~3 μm in diameter, and some displayed 'zig-zag' arrays of cristae (Daniels & Breyer, 1968). The cell surface comprised a trilaminar plasma membrane and an external filamentous cell coat. Different types of vesicles and vacuoles were present (Stockem, 1969; Flickinger, 1973a) including food vacuoles, fringed vacuoles with a membrane and lining similar to the cell surface, groups of small vesicles, and crystal-containing vacuoles. The nuclear envelope consisted of 2 membranes and a prominent fibrous lamina, which had a honeycomb-like configuration in *A. discoides*. The interiors of the nuclei in both species contained chromatin and multiple nucleoli. Further details of normal amoeba ultrastructure are found in the articles and reviews.
Cytoplasmic injection in amoebae

Amoebae injected with heterologous cytoplasm. At the earliest intervals, 15 min and 2 h after injection of heterologous cytoplasm from Pelomyxa carolinensis, few ultrastructural changes were observed in recipient *Amoeba discoides*, although the cytoplasm did contain some large mitochondria, reminiscent of those of normal *P. carolinensis*. At these and subsequent intervals up to 1 d, a small proportion (~1–2%) of mitochondria had a matrix of decreased density or disruption of the bounding membranes, and clumps of a flocculent dense material were observed in the cytoplasmic matrix (Fig. 1).

The most prominent change in cells fixed 6 or 12 h after injection was the presence of structurally abnormal organelles within membrane-bounded spaces in the cytoplasm (Figs. 2–4). Organelles segregated thus from the remainder of the cytoplasm included mitochondria, pieces of endoplasmic reticulum and fringed vacuoles. The enclosed organelles had disrupted membranes, their normal contours were distorted, and some were unusually dense. Accompanying recognizable organelles were vesicles, granules, and a flocculent, dense material, but much of the interior of the vacuole-like spaces was empty of formed material. Donor nuclei were generally too large to enter the micropipette used to transfer cytoplasm, but in rare instances fragments of nuclear envelope were encountered in the membrane-bounded structures. In a few cases the vacuoles contained pieces of plasma membrane (Fig. 2). Most organelles in the general cytoplasm outside the membrane-delimited spaces were not altered. A few distorted mitochondria constituted exceptions to this generalization, and in some cells cisternae of the rough endoplasmic reticulum appeared to encircle parts of the cytoplasm.

When the localization of acid phosphatase was examined, electron-dense reaction product was observed in the membrane-bounded areas of recipient cells along with altered organelles (Fig. 4). Additional sites of acid phosphatase activity included cisternae and vesicles at the convex pole of the Golgi apparatus and lysosomes of variable size (Wise & Flickinger, 1970). Electron-dense precipitate was not observed in these locations in samples of control cells incubated in medium lacking substrate.

The altered organelles separated from the remainder of the cytoplasm by a membrane were initially thought to be from the injected heterologous cytoplasm because similar structures were not observed in normal uninjected amoebae from the same cultures or in those injected with fluid rather than cytoplasm (Flickinger, 1974a). The presence in some instances of pieces of plasma membrane or nuclear envelope also indicated the exogenous origin of the contents. However, the possibility was considered that the membrane-encircled regions might be formed by autophagy of host cell organelles if this were somehow induced by the injection. To aid in distinguishing between these 2 alternatives donor cytoplasm was marked with thoria particles. Donor amoebae that had been exposed to thoria solution contained electron-dense particles of thoria in cytoplasmic vesicles of varying size (Fig. 5), the thoria evidently having been taken into the cell by endocytosis. Recipient amoebae fixed 6 h following the injection of thoria-containing cytoplasm contained electron-dense particles identified as thoria in the membrane-bounded structures (Fig. 6) along with
Fig. 1. In recipients of heterologous cytoplasm, a few mitochondria (m) have an electron-lucent matrix and an irregular outline. Patches of a flocculent dense material (d) are present in the cytoplasmic matrix. Amoeba 6 h after injection. x 30,500.

Fig. 2. A membrane (a) separates a variety of organelles from the general cytoplasm (c) of a recipient amoeba. Identifiable structures thus enclosed include distorted mitochondria (m), filaments, vesicles, and pieces of plasma membrane (p) identified by the presence of filamentous cell coat material. Injected cell 12 h after operation. x 16,000.
degenerating organelles and debris, indicating that the membrane-bounded regions contained injected cytoplasm. Concentrated deposits of thoria were also observed in some vesicles outside the membrane-delimited spaces at 6 h, which suggests that not all the injected cytoplasm had been segregated from that of the host by this time after the operation. Thoria was not observed in large vacuoles identified as old food vacuoles on the basis of their size, polymorphous content filling most of the interior and presence in uninjected cells. It may be noted also that, since recipient amoebae were starved for at least 1 day and usually for 2 days prior to use, organelles of food organisms were no longer recognizable by the time an injection of cytoplasm was performed.

In recipient cells prepared 1 day after injection of *P. carolinensis* cytoplasm, irregular vacuole-like structures containing apparently degenerating material were also present (Fig. 7). Definite identification of organelles within membrane-bound structures was more difficult than at earlier intervals, however, and by 2 or 3 days after operation the density and degree of disruption of the contents were so great that it was rarely possible to recognize organelles in the contents of vacuoles. The remaining cytoplasmic organelles of the host did not appear altered in samples fixed 1–3 days after injection (Fig. 8).

No changes from normal were detected in nuclei of host cells, including the chromatin, nucleoli, and nuclear envelope.

**Amoebae injected with homologous cytoplasm.** Amoebae injected with homologous cytoplasm showed few differences from normal. At intervals of 6–12 h after operation, a small number of vacuole-like structures enclosing apparently degenerating organelles were encountered. These structures were uncommon in cells injected with homologous cytoplasm compared to the frequent examples observed when heterologous cytoplasm was transferred, as illustrated by the results of the following semiquantitative procedure. All the profiles of amoebae on a grid were scanned for the presence of vacuole-like spaces containing organelles. In a sample of amoebae that had received heterologous cytoplasm 6 h previously, 46% (13/28) profiles contained membrane-bounded degenerating organelles, while in a comparable sample of cells that received homologous cytoplasm only 16% (4/25) contained these structures (*P* < 0.025). Furthermore, the profiles of cells that had been injected with heterologous cytoplasm often displayed multiple large vacuole-like areas while those of the homologous recipients had only one or a few examples. Uninjected cells from the culture from which recipients were obtained lacked similar structures.

**DISCUSSION**

Following the introduction of heterologous cytoplasm, 2 main responses by the host amoebae were observed. First, some of the injected cytoplasm which did not disperse throughout that of the recipient was rapidly expelled. This was shown by direct continuous observation of living cells by light microscopy. Secondly, organelles were segregated from the remainder of the host cytoplasm by a membrane and under-
went degeneration within vacuole-like spaces. The presence of acid phosphatase reaction product indicated that the segregated organelles underwent digestion by lysosomal enzymes. Ultrastructural observations suggested that injected organelles were involved, and the use of an electron-opaque marker confirmed the presence of donor cytoplasm within the vacuoles. The contention that heterologous organelles were segregated and digested in this way is in accord with the previous report that when materials such as bacteria, spores, polystyrene and carbon particles are injected into an amoeba they become surrounded by a membrane and thus are enclosed within vacuoles, which subsequently acquire acid phosphatase activity (Casley-Smith & Savanat, 1966). The endosymbionts that infect amoebae are also surrounded by a membrane, although in contrast to injected materials symbiont-containing vacuoles lack acid phosphatase activity (Jeon & Jeon, 1976).

The observations suggested that the responses of the recipient cells were related to the heterologous nature of the injected cytoplasm. First, damage to cytoplasm during manipulation did not appear to account for the responses, because changes of the same magnitude were not seen when homologous cytoplasm was injected. In particular, segregation and degeneration of organelles when heterologous cytoplasm was injected greatly exceeded that observed when homologous cytoplasm was transferred. A low level of degeneration of organelles was detected after the injection of homologous cytoplasm, however, and this may be related to damage to a small fraction of organelles during the operation. Secondly, the responses to heterologous cytoplasm were not due merely to the introduction of material of a certain bulk because injection of comparable amounts of distilled water or aqueous solutions of radioactive precursors (Flickinger, 1974a), as well as homologous cytoplasm, did not produce similar changes.

These considerations suggest that amoebae are able to distinguish heterologous from homologous cytoplasmic organelles, although the mechanism of this postulated recognition is unknown at present. How the apparent rejection of heterologous cytoplasm is related to the lethal antimitotic factors and incompatibilities between nucleus and cytoplasm of different strains of amoebae (Jeon & Danielli, 1971; Jeon & Lorch, 1970; Cameron & Hawkins, 1976) is also not known, but different mechanisms may be involved because even incompatible nuclei are not segregated in hybrid amoebae, and some appear to function for days before the hybrid cells die (Jeon & Danielli, 1971; Flickinger, 1973b).

Fig. 3. Vacuoles in the cytoplasm of amoeba injected with heterologous cytoplasm have various contents, including altered but recognizable organelles. In these and other illustrations, attention is directed toward mitochondria (m) in the vacuoles because even when structural changes have occurred their distinctive morphology makes them more readily recognizable than most other organelles. Amoebae 12 h after injection. × 23000.

Fig. 4. Acid phosphatase reaction; amoeba 6 h after injection of heterologous cytoplasm. An irregularly contoured membrane encloses debris, an altered but recognizable mitochondrion (m), and copious electron-dense reaction product (r). × 22000.
Cytoplasmic injection in amoebae
Fig. 5. Cytoplasm of a donor *P. carolinensis* exposed to a thoria solution for 30 min. Electron-dense particles of thoria (arrow) are present in vesicles and vacuoles. × 21,000.

Fig. 6. Recipient amoeba 6 h after injection of cytoplasm from a thoria-containing donor cell. A membrane-bound space contains electron-dense particles identified as thoria (arrows) as well as a remnant of a mitochondrion (h), vesicles, and a flocculent dense material. Some of the membranes (b) have granules resembling ribosomes on their surfaces and thus may derive from endoplasmic reticulum. × 30,000.
Fig. 7. Portion of a recipient amoeba 24 h after injection of heterologous cytoplasm. Identification of organelles in vacuoles is difficult by this stage after injection. However, in this example the debris may include the remnants of a mitochondrion (h). x 18000.

Fig. 8. The bulk of the cytoplasm outside of membrane-enclosed areas in recipients of heterologous cytoplasm appears normal. er, rough endoplasmic reticulum; f, fringed vacuole; g, Golgi apparatus; m, mitochondrion; s, cell surface. Recipient amoeba 2 days after operation. x 18000.
A relatively long-term response to injection of heterologous cytoplasm was a sharp decrease in viability approximately 1 week after the operation, which confirms previous observations that heterologous cytoplasm has a lethal effect on the recipient (Hawkins & Cole, 1965; Jeon & Lorch, 1970, 1971; Cameron & Hawkins, 1976). In the present study, the decline followed a period of improvement in the gross morphology of the recipient amoebae between 3 and 6 days after the operation. It may be speculated that this transient improvement was related to the successful segregation of the heterologous organelles. Nevertheless, the host cells subsequently died, presumably due to the action of the lethal cytoplasmic factor (Jeon & Lorch, 1970; Cameron & Hawkins, 1976), which apparently was not destroyed, or exerted an irreversible effect at an earlier stage.

Among the segregated organelles identified in injected cells were mitochondria, endoplasmic reticulum and filaments. Pinocytic vesicles or vacuoles were evidently included also, because they were the carriers of thoria when the donor cytoplasm was marked with this substance. Other structures might have been present but were so distorted that they were not recognized. Thus it is not clear whether the host cells exercise any selectivity in their response to different types of organelles in injected cytoplasm.

The source of the membrane that surrounded and segregated organelles in injected cells is of interest. The observation that parts of the cytoplasm were encircled by cisternae of the rough endoplasmic reticulum in cells injected with heterologous cytoplasm may indicate that these membranes of the host are involved. It should be noted, however, that similar configurations have been observed in amoebae under a variety of deleterious conditions, including enucleation (Flickinger, 1968, 1973a), and may simply reflect an adverse effect of the foreign cytoplasm.

The responses of amoebae to heterologous cytoplasm have some similarities to the reactions of HeLa cells to the injection of foreign proteins. When various rhodamine-conjugated proteins were introduced by microinjection (Stacey & Allfrey, 1977), the proteins became sequestered in parts of the cytoplasm. This may be analogous to the segregation of injected organelles within membrane-bound structures in injected amoebae. Furthermore, the half-time for segregation of different proteins in HeLa cells ranged from less than 3 h to about 3 days, which is generally comparable to the time required for segregation of injected organelles into vacuoles in amoebae. Interestingly, when HeLa cells were injected with large amounts of highly concentrated protein solutions, the mass of segregated protein was extruded from the cell. This may be analogous to the extrusion of some cytoplasm observed in the present study.

This research was supported by grants from the American Cancer Society (VC-169A, B, C; CD-58D). The assistance of Mrs Christine Folsom-Kovarik and Miss Kathleen Coogan with parts of the work is gratefully acknowledged.
REFERENCES


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(Received 21 February 1980 – Revised 14 April 1980)